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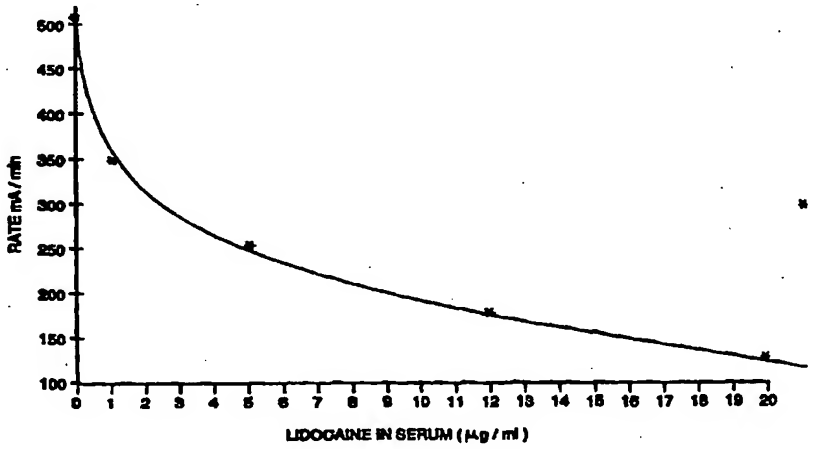

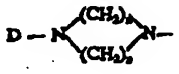

(a)

(57) Abstract

The invention provides a reactive derivative of dialkyl amino compounds, particularly dialkyl amino drugs, for facilitating the conjugation of the drug, directly or through a bifunctional spacer, to a carrier compound such as proteinaceous materials. The derivative has formula (a) wherein D is the drug and n is an integer greater than 1, and preferably equal to 2. The drug derivative carrier conjugate can be used as an immunogen for production of antibodies specific to the drug. Additionally, the conjugate can be coupled to a solid support, such as a polymer particle, for use as a particle reagent in immunoassays specific to the drug.

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TITLE OF INVENTION

PREPARATION OF IMMUNOGENS AND OTHER CONJUGATES OF DRUGS

Field of the Invention

The present invention relates to the use of piperazine derivatives to prepare immunogens and other conjugates of drugs having dialkyl amino groups.

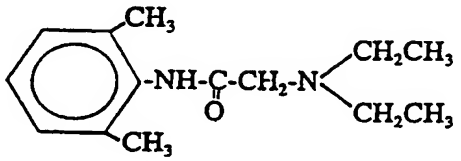
Description of the Invention Background

Individuals receiving drug therapy often must be regularly monitored for the levels of the drug in that patient's blood serum or other body fluids. Assays for detecting the levels of certain drugs are available. For example, the blood serum level of lidocaine, an antiarrhythmic, may be detected by gas chromatography, high performance liquid chromatography or enzyme immunoassays.

A highly sensitive immunoassay is provided by light scattering immunoassays that use particle reagents having high refractive indices, such as the particle enhanced turbidimetric inhibition immunoassays described in U.S. Patent Nos. 4,401,765 and 4,480,042. Automated clinical analyzers, such as the DuPont aca automated clinical analyzer, automatically mix reagents from a single dose, prepackaged test pack with a patient sample and incubate the sample and reagents at the desired temperature, 37°C, for a desired time, then read and print the results. Such automated analyzers have become essential in clinical laboratories because of their accuracy, reproducability of results and ease of use. Light scattering immunoassays, heterogenous immunoassays, affinity column mediated immunoassays, and some enzyme immunoassays may all be performed on the automated analyzers. A particle reagent or some other solid support bound to a reactive derivative of the analyte of interest and an antibody specific to the analyte must be available for use in these sensitive assays.

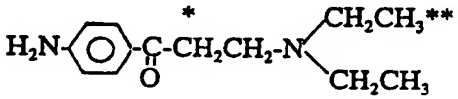
Heretofore, lidocaine particle reagents suitable for use in automated analyzers such as those described have not been available. The gas and high performance liquid chromatography methods of detection are not adapted for use in such automated analyzers.

Lidocaine includes a dialkyl amino group in its structure,



It is believed that particle reagents suitable for use in automated analyzers are also not available for many other drugs having dialkyl amino groups.

Procainamide is another drug having a dialkyl amino group in its structure,



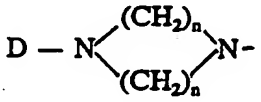
In one known reagent, a protein is bound at the methyl site designated by *; in another, a protein is bound at the site designated by ** above.

Methods for the development of antibodies to the drugs imipramine and amitriptyline, both of which have dialkyl amino groups in their structures, have been described in the literature. In both cases the structures were modified by attachment of spacer arms to the aromatic ring of the compound. The spacer arms were then attached to bovine serum albumin and used to produce the antibodies. *See*, Adamczyk, M. et al., J. Immunol. Methods, vol. 162(1), pp.47-58 (1993) and vol. 163(2), pp.187-97 (1993).

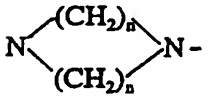
There is a need for a means of facilitating the conjugation of dialkyl amino drugs to proteins to provide immunogens and particle reagents for use in the more sensitive, rapid and reliable automated analysis techniques available. There is a specific need for reactive derivatives of dialkyl amino drugs for the preparation of immunogens and antigens for suitable for use in the commercially available automated analyzers for quantitating lidocaine and other dialkyl amino drugs in human serum.

SUMMARY OF THE INVENTION

The objects of the invention are satisfied by a composition for conjugation to a carrier compound, wherein the composition has the following formula:



wherein n is an integer greater than or equal to 1 and D is a compound, preferably a drug, having in its underivatized structure a dialkyl amino group. In the preparation of the composition of the present invention, the dialkyl amino group of the compound is replaced with the



residue, a piperazine like derivative which substantially preserves the structure of the compound D while adding a reactive terminal nitrogen for conjugation to a carrier compound.

Optionally, a bifunctional spacer can be bound to the terminal nitrogen to link the reactive derivatized composition of the present invention to the carrier compound. The bifunctional spacer may be selected from the group consisting of cycloanhydrides, bis-N-succinimidyl derivatives and aldehydes.

The carrier compound may be (i) a proteinaceous material, such as keyhole limpet hemocyanin, ovalbumin or bovine serum albumin (BSA), or (ii) a synthetic polymeric material, such as polyethylene polyamine or polyethylene glycol. The derivatized composition - carrier compound conjugate can be used as an immunogen for the production of antibodies specific to the compound D, or may be bound, through the carrier compound, to a solid support, such as a polymer particle, for use as a reagent in immunoassays for the detection of levels of the compound D.

A preferred particle reagent of the present invention may include a polymer particle having an inner core and an outer shell wherein the inner core is a polymer having a refractive index of not less than 1.54 as measured at the

wavelength of the sodium D line. The outer shell is a polymer of (i) an ethylenically unsaturated monomer having a functional group capable of reacting with a nucleophilic compound of biological interest, (ii) optionally, other ethylenically unsaturated monomers in an amount sufficient to produce water insoluble polymer particles, and (iii) not more than 10 parts by weight of the outer shell of the monomers of the inner core, the outer shell being formed by polymerization in the presence of the inner core covalently bound to the derivatized composition - carrier compound conjugate described above.

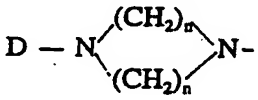
DESCRIPTION OF THE DRAWINGS

The success of the methodology of the present invention is illustrated in the Figure which shows a graph of the rate of agglutination as measured by the change in miniabsorbance at 340nm per minute (mA/min.) against the concentration of lidocaine in the sample.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a novel means for facilitating the conjugation of compounds, in particular, drugs having dialkyl amino groups, to carrier compounds by replacing the dialkyl amino group with piperazine. The carrier compounds can be proteinaceous materials, such as antigens or enzymes. The additional amino group provided by the derivatization to a piperazine-containing structure facilitates the conjugation. If desired, a spacer can be positioned between the terminal amino group of the piperazine and the carrier.

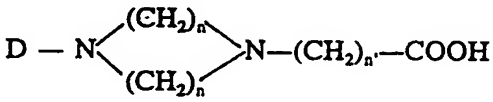
The invention provides a reactive derivative of dialkyl amino drugs having the following structure:



wherein D is any drug which, in its underivatized structure, normally contains a dialkyl amino group and n is an integer equal to or greater than 1, and preferably between 1 and 3, and most preferably equal to 2. The n number for each of the dialkyl chains of the reactive derivative of the invention is equal.

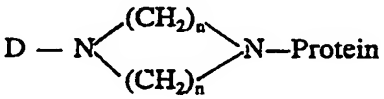
The protein does not bind to the dialkyl chain and the binding is not affected by the length of the chain, so the length of the chain is not critical to the function of the piperazine derivative or the conjugate. Moreover, changing the length of the dialkyl chain in the original drug to a piperazine like structure will not alter the recognition of antibodies to the drug.

Alternatively, the composition may include a bifunctional spacer to link the reactive drug piperazine derivative to the proteinaceous carrier compound, as shown in the structure below;

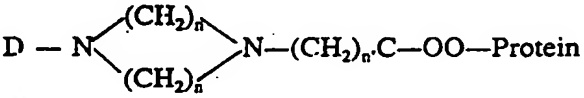


wherein n' is an integer from 0 to 6. Other bifunctional spacers may be used also. For example, amino group directed bifunctional groups such as cycloanhydrides, bis-N-succinimidyl derivatives and dialdehydes may serve as the bifunctional spacer. Many other possible bifunctional spacers can be used. *See*, for example, the homobifunctional spacers listed in Table 1 on pages 76 - 97 of Chapter 4 and the heterobifunctional spacers listed in Table 1 on pages 152 - 167 of Chapter 5 of S.S. Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING, (CRC Press 1991).

To use the reactive drug derivative as an antigen, a protein, for example, is attached to the terminal nitrogen or to the functional group of the bifunctional spacer, as follows:



or

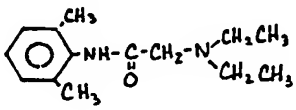
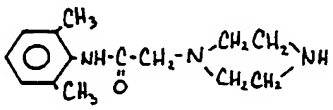
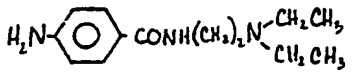
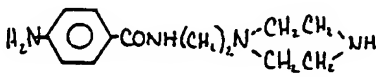


Alternatively, the drug derivative-protein conjugate, with or without a spacer, can be used to immobilize drug on a solid support, such as the particle reagents

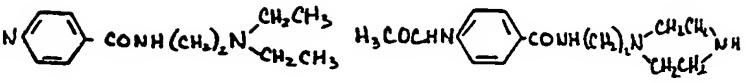
described in U.S. Patents Nos. 4,401,765 and 4,480,042, the disclosures of which are incorporated herein by reference, or any suitable solid support surface. The drug derivative-carrier conjugates of the invention are particularly suitable for use in the immunoassays conducted in the conventional automated clinical analyzers discussed above. There are many different proteins that can be used for conjugation. The choice will depend on the ultimate application of the drug-protein conjugate. For example, if the conjugate is to be used to attach the drug to a solid support or to a particle for use as an antigen in an immunoassay, a linker, such as polyethylene polyamine (PEPA) and human serum albumin (HSA) or bovine serum albumin (BSA) may be used. The same conjugate can be used as an immunogen. However, for reasons of immunogenicity and specificity, a different protein carrier is preferred. If the conjugate will be used as an immunogen, BSA, ovalbumin or keyhole limpet hemocyanin (KLH) are preferred. The various proteinaceous carriers are conjugated to the reactive drug piperazine derivative by known techniques, as described in S.S. Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING, (CRC Press 1991).

Examples of dialkyl amino drugs which can benefit from the piperazine derivatization of the present invention are shown in Table I below.

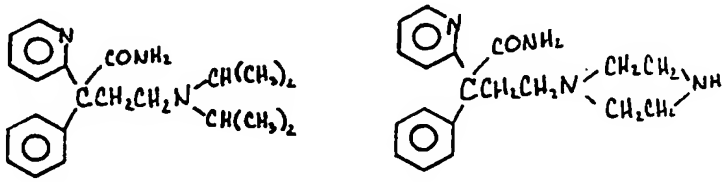
TABLE I

Drug		Piperazine Derivatized Drug
Name	Structure	Structure
Lidocaine (local anesthetic; antiarrhythmic)		
Procainamide (antiarrhythmic)		

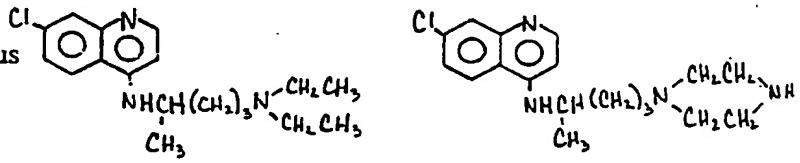
N-acetyl procainamide (antiarrhythmic)



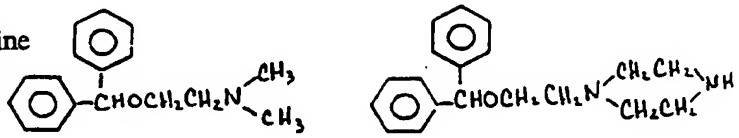
Dispyramide (antiarrhythmic)



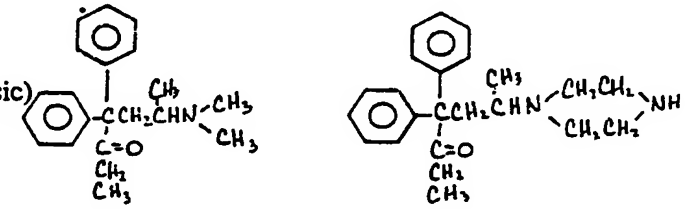
Chloroquine (antimalarial, antiamebic, lupus suppressant)



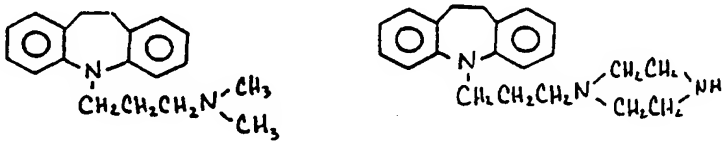
Diphenhydramine (antihistamine)



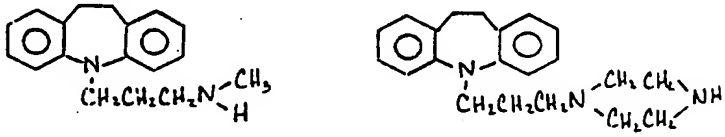
Methadone (narcotic analgesic)



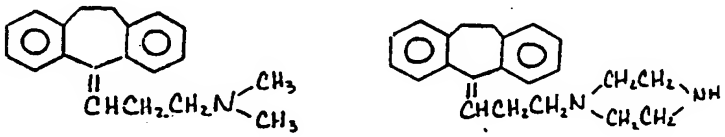
Imipramine (antidepressant)



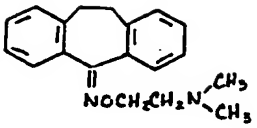
Desipramine (antidepressant)

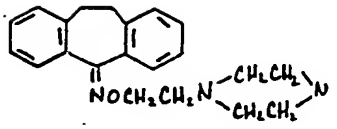


Amitriptyline (antidepressant)

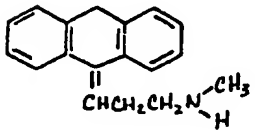


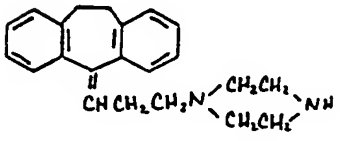
Noxiptilin
(antidepressant)





Nortriptyline
(antidepressant)





The reactive drug derivatives of the invention, when used as immunogens, produce an antibody that is more specific to the basic drug than is possible with prior art attempts to develop antibodies to certain of these drugs. Because the structure of piperazine is so very close to the structure of the dialkyl amino groups, the piperazine derivatives preserve the basic drug structure.

The resulting compound differs from the basic structure of the drug only in the addition of the amino which, in effect cyclizes the dialkyl amino branches which had been present on the basic drug structure. The prior art techniques couple a protein at a site in the drug structure that alters that basic structure.

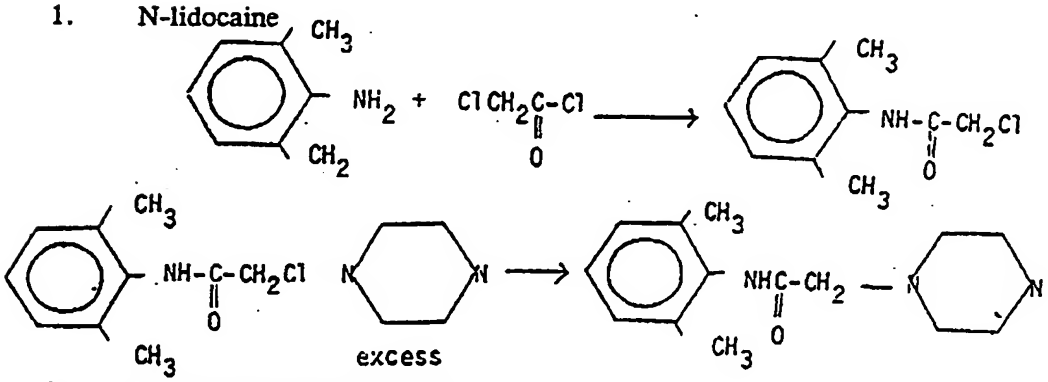
The amino of piperazine is used to couple the drug to protein by known techniques to produce the immunogen. (*see*, S.S. Wong, *supra*.) Thus, the structure of the drug is preserved without significant alteration and the attachment is at a site which does not interfere with the drug.

Examples demonstrating the preparation and use of the piperazine derivative of dialkyl amino drugs of the present invention follow.

The following lidocaine derivatives have been synthesized and characterized: N-lidocaine and N-lidocaine propionic acid.

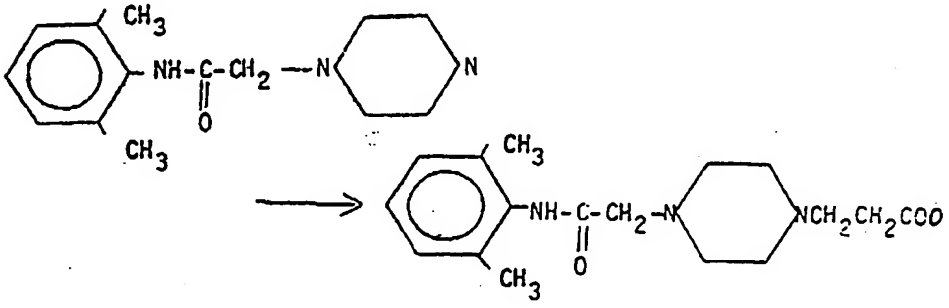
LIDOCAINE DERIVATIVES FOR IMMUNOGEN AND PARTICLE REAGENT SYNTHESIS

1. N-lidocaine



excess

2. N-lidocaine-propionic acid



The following immunogens have been prepared: N-lidocaine-BSA, N-lidocaine-KLH, N-lidocaine-propionic acid-BSA and N-lidocaine-propionic acid-KLH. KLH is known to be a better immunogen, but its low solubility makes it difficult to handle and characterize. Thus, BSA conjugates were also prepared as back-up. These immunogens are ready for use to initiate lidocaine antibody production by known techniques.

The proper choice of a reactive lidocaine derivative for the immunogen and particle reagent preparation is critical because, otherwise, the immunogen will not generate antibodies specific to lidocaine and the particle reagents will not specifically bind lidocaine in the patient serum.

Lidocaine particle reagents have been prepared from N-lidocaine-HSA, N-lidocaine-PEPA 1300 (Polyethylene polyamine, average molecular weight 1300), N-lidocaine-propionic acid-HSA and N-lidocaine-propionic acid-PEPA

200. When tested with a goat anti-lidocaine from Kallestad Laboratories, all particle reagents showed high activity in a Cary 19 spectrophotometer. 12μl of particle reagent and 8μl of anti-lidocaine were incubated using 2.5% polyethylene glycol 800 (PEG), 0.1% sodium dodecyl sulfate (SDS) in 0.15M phosphate (pH 7.8) as the assay buffer. Either anti-HSA activity or no activity was observed when the particle reagent were tested with several lots of rabbit anti-lidocaine.

A lidocaine particle enhanced turbidimetric inhibition immunoassay curve was demonstrated for the N-lidocaine-HSA-particle reagent and the results are shown in the Figure. The rate of agglutination as measured by the change in miniabsorbance at 340nm per minute vs. the drug concentration of the sample was plotted. The presence of the drug in the sample inhibits agglutination, so the higher the drug concentration, the lower the agglutination rate. The assay was not optimized, but good separation was achieved in the therapeutic range. The results indicated that the lidocaine particle reagent and immunogen are suited for lidocaine particle enhanced turbidimetric inhibition immunoassay. Further, the therapeutic range for lidocaine (1.0-12μg/μl) is well within the sensitivity range of the particle enhanced turbidimetric inhibition immunoassay (about 0.5μg/ml - 50μg/ml).

EXAMPLES

Example 1: Lidocaine Derivatives Syntheses

a. Preparation of N-lidocaine

1. N-chloroacetyl-2, 6-xylidine. A solution of 24 mL of 2, 6-xylidine (0.2 mole) in 160 mL of glacial acetic acid was cooled on ice to 10°C and 17.1 mL of chloroacetyl chloride (0.22 mole) was added all at once. The mixture was stirred vigorously for 10 min. and 200 mL of half-saturated sodium acetate solution was added at one time. White precipitate formed immediately. The mixture was made up to 500 mL with water and stirred at room temperature for 30 min., then at 4°C for an hour. The white powder was collected by filtration and recrystallized in aqueous methanol to give white fine needle crystals, m.p. 145-146°C, dry weight 32.5 g (83%).

2. Preparation of N-lidocaine. A sample of piperazine (17.2 g, 0.2 mole) was dissolved in 150 mL of ethyl acetate with heating. To the hot solution was added 3.98 g of N-chloroacetyl-2, 6-xylidine (0.02 mole) from step b.1 above in 50 mL of ethyl acetate and the mixture was refluxed for 30 min. After being cooled on ice for 30 min., the solution was filtered to remove the piperazine hydrochloride formed. The filtrate was washed three times with 20 mL portions of water and dried with anhydrous sodium sulfate. The solvent was removed in a rotary evaporator at 70°C for one hour. The oily residue solidified on cooling. It was collected and dried under vacuum. The dried precipitate had the following properties: m.p. 109-114°C, weight 3.82 g (77%), NMR (deuterated chloroform): ppm downfield from tetramethyl silane; δ 2.2 (6H, singlet), δ 2.6 (4H, triplet), δ 2.9 (4H, triplet), δ 3.1 (2H, singlet), and δ 7.0 (3H, singlet).

b. Preparation of N-lidocaine-propionic acid. A mixture of 1.23 g of N-lidocaine (5 mole) from step b.2 above and 1.25 g of iodopropionic acid (6.25 mmole) in 20 mL of acetonitrile was heated at 70°C for one hour. To the mixture was added 1.25 mL of triethyl amine and heating was continued for another 30 min. The mixture was cooled on ice for 30 min. The precipitate was collected on filtration, washed with cold acetonitrile and dried. The dried precipitate had the following properties: m.p. 250-252°C, weight 1.5 g (94%), NMR (deuterated trifluoroacetic acid): ppm downfield from tetramethyl silane; δ 2.2 (6H, singlet), δ 3.2 (2H, triplet), δ 3.85 (2H, triplet), δ 4.2 (8H, board singlet), δ 4.7 (2H, singlet), and δ 7.1 (3H, singlet).

Example 2. Lidocaine Conjugates Syntheses

a. Preparation of N-lidocaine-protein conjugates

1. To a solution of 300 mg HSA (purified to the fifth fraction, i.e., fraction V) in 17 mL of water was added 300 mg of ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDPC). The pH was adjusted to 6 (by the addition of 0.1M hydrochloric acid) and 150 mg of N-lidocaine in 3 mL of ethyl alcohol was added. The pH was readjusted to 6. The mixture was stirred at room temperature for 90 min. and another 150 mg

of EDPC was added. The mixture was then stirred at 4°C overnight and dialyzed exhaustively against 15mM phosphate buffer (pH 7.8).

2. The same procedure was used for the preparations of N-lidocaine-BSA and N-lidocaine-KLH conjugates.

b. Preparation of N-lidocaine-propionic acid conjugates

1. A solution of 160 mg of N-lidocaine-propionic acid (0.5 mmole) in 10 mL of dimethyl formamide (dried over molecular sieves) was cooled on ice and 139 μL of triethyl amine (0.5 mmole) was added followed by 130 μL of isobutyl chloroformate (0.5 mmole). The mixture was stirred at 4°C for 20 min. and added to 15 mL of water containing 300 mg of PEPA-200. The mixture was kept at 4°C for 30 min. and then at room temperature for 5 hours. The product was used without dialysis.

2. The same procedure as the preparation of PEPA-200 conjugate was used except the products were dialyzed against 15mM phosphate buffer (pH 7.8).

Example 3: Preparation of Lidocaine Particle Reagents (PR)

a. N-lidocaine-HSA-PR A solution of 1 mg/mL N-lidocaine-HSA, 0.4% particle raw material and 0.18% of GAFAC in 34 mL of 15mM phosphate buffer (pH 7.5) was heated at 70°C for 45 min. The mixture was cooled on ice and centrifuged in a Sorvall RC-5B centrifuge at 19k rpm for 90 min. The supernatant was discarded and the pellet was resuspended in 15 mL of 15mM phosphate buffer. The particle reagent was centrifuged again and the pellet resuspended in 5 mL 15mM glycine (pH 9.0) and 0.01% thimerosal by sonication.

b. N-lidocaine-PEPA-PR, N-lidocaine-propionic acid-HSA-PR and N-lidocaine-propionic acid-PEPA-PR The same procedure was used for the preparation of other lidocaine particle reagents except pH 9.5 was used for PEPA-particle reagent syntheses.

Example 4: Particle Enhanced Turbidimetric Inhibition Immunoassay (PETINIA) Reactivity

PETINIA reactivity of the lidocaine particle reagents was measured in a one mL solution of 150mM phosphate buffer containing 2.5% PEG and 0.1% SDS, 8 μL of anti-lidocaine, 10 μL of calibrator and 12 μL of particle reagent. The reaction rate was followed at 340nm in a Cary 19 spectrometer at room temperature.

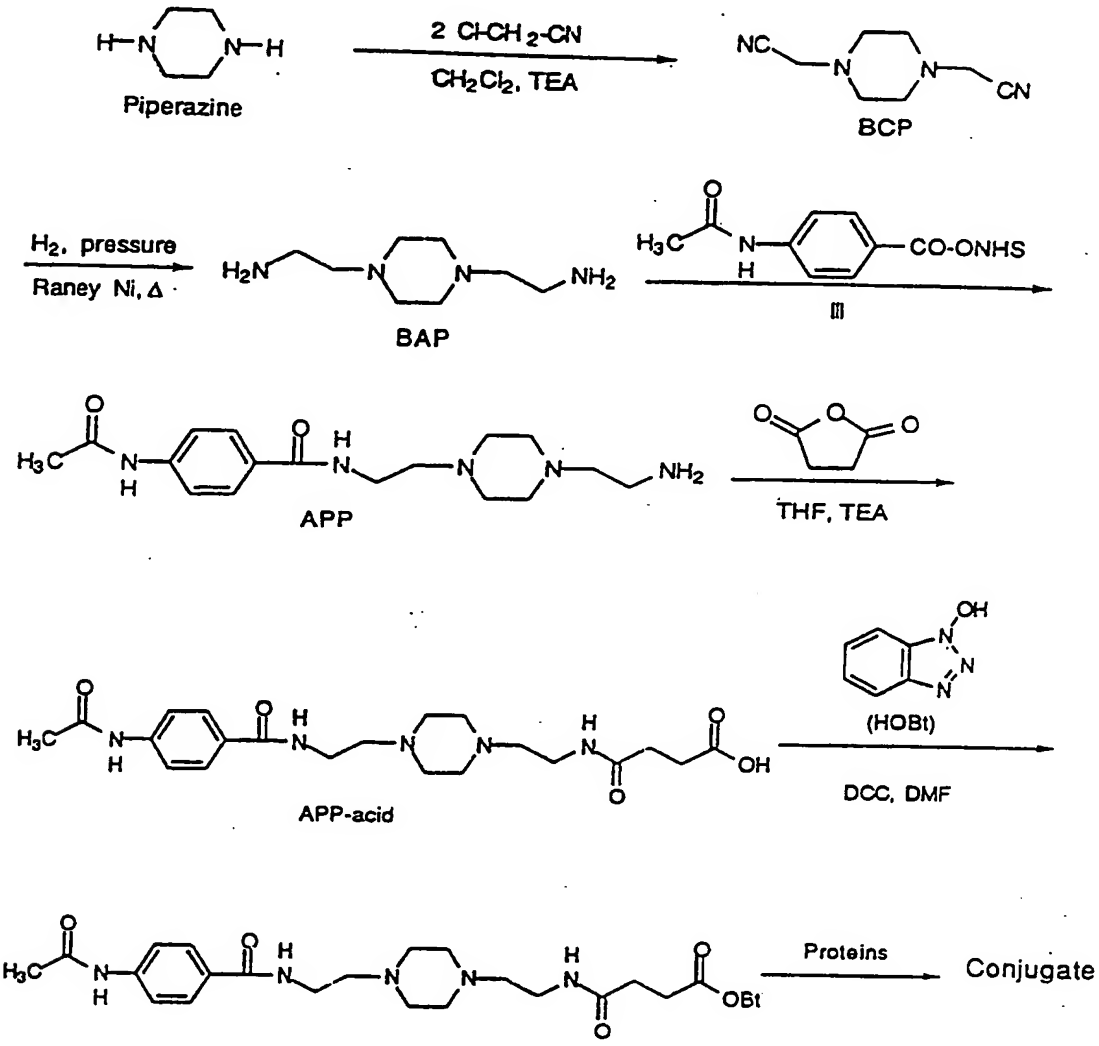
Several lots of particle reagent, when tested with rabbit anti-lidocaine, gave rates of approximately 400 mA/min, but the reaction was not inhibitable by lidocaine. When tested with goat anti-lidocaine, the same rates were obtained and were 80% inhibitable by lidocaine. The results are shown in Table II.

TABLE II

REACTIVITY OF LIDOCAINE - PARTICLE REAGENTS

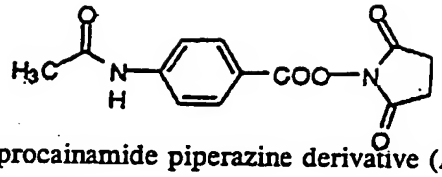
LIDOCAINE PARTICLE REAGENT (PR)	ANTILIDOCAINE	CALIBRATOR USED (μg/mL)	RATE mA/min
N-lidocaine-HSA-PR	rabbit	0	600
N-lidocaine-HSA-PR	rabbit	12	540
N-lidocaine-propionic acid-PEPA-PR	rabbit	0	400
N-lidocaine-propionic acid-PEPA-PR	rabbit	12	380
N-lidocaine-PEPA-PR	rabbit	0	360
N-lidocaine-PEPA-PR	rabbit	12	330
N-lidocaine-HSA-PR	goat	0	510
N-lidocaine-HSA-PR	goat	1	350
N-lidocaine-HSA-PR	goat	5	250
N-lidocaine-HSA-PR	goat	12	175
N-lidocaine-HSA-PR	goat	20	125
N-lidocaine-propionic acid-PEPA-PR	goat	0	360
N-lidocaine-propionic acid-PEPA-PR	goat	1	270
N-lidocaine-PEPA-PR	goat	0	370
N-lidocaine-PEPA-PR	goat	1	270

A sequence for the derivatization of N-acetyl procainamide (NAPA) or procainamide follows.

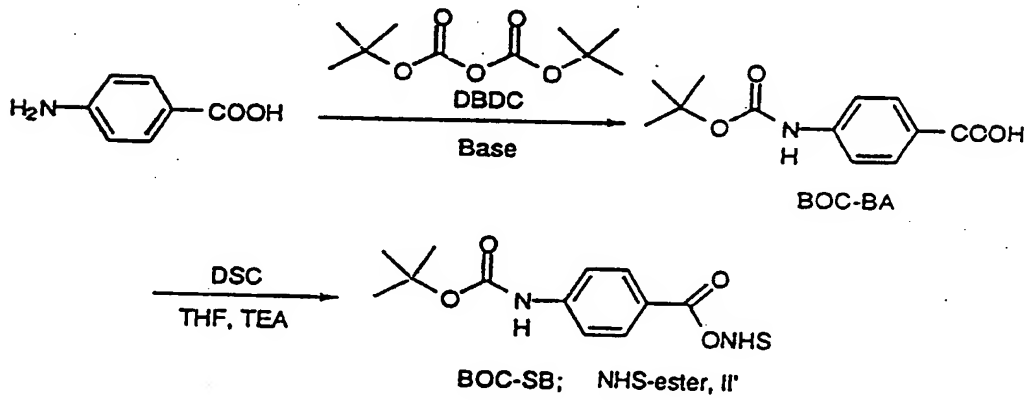


I

A key step in the process of making a piperazine derivative for procainamide and NAPA is to make bis (aminoethyl) piperazine (BAP). As shown in the reaction sequence I above, piperazine was used to react with chloroacetyl nitrile to afford bis (cyanomethyl) piperazine (BCP), and BCP was reduced by catalytic hydrogenation under pressure to generate bis (aminoethyl) piperazine (BAP). BAP was coupled with NHS-ester (II)

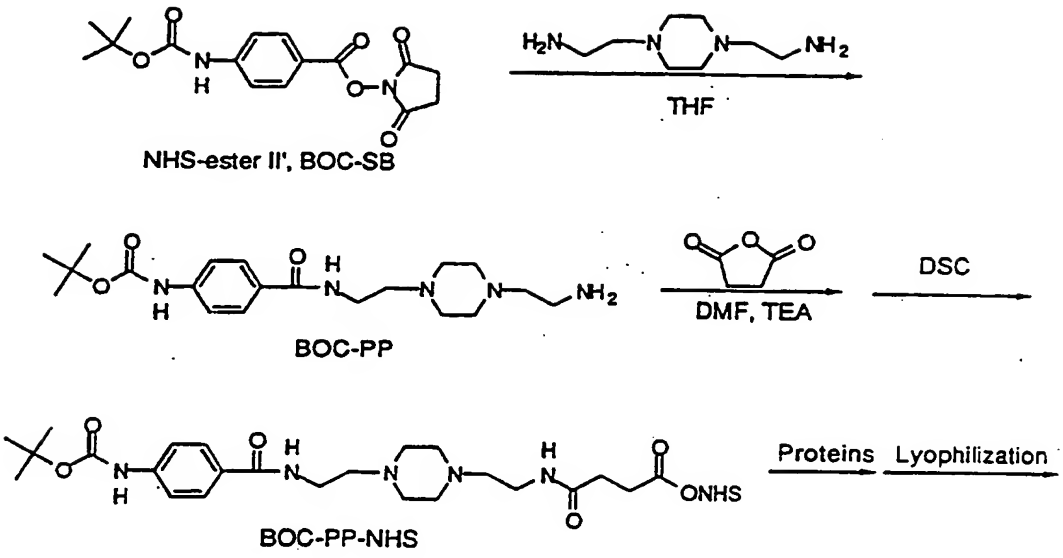


to form the acetylprocainamide piperazine derivative (APP, NAPA piperazine derivative). The protein conjugate linker was introduced by reacting APP with succinic anhydride, and then it was coupled with proteins to afford NAPA protein conjugates. The synthesis of procainamide protein conjugates involved one more protecting and deprotecting steps as compared to the one of NAPA. As shown in the reaction sequence II below, a t-butoxy carbonyl (t-BOC) group was used to protect the amino group of p-amino benzoic acid to form t-butoxylcarbamyl benzoic acid (BOC-BA), then BOC-BA reacted with disuccinimidyl carbonate (DSC) in tetrahydrofuran (THF) and triethyl amine (TEA) solution to generate NHS-ester II', t-butoxyl carbamyl succinimidyl benzoate (BOC-SB).



II

The BOC protecting group was removed in organic solution (dichloromethane) with trifluoroacetic acid after procainamide was conjugated to proteins as shown in the reaction sequence III.



Using the BAP derivative approach described above, two NAPA and two procainamide protein conjugates (with ovalbumin and KLH) were synthesized. The limitation for this approach is the high pressure hydrogenation of BCP, which resulted in a low yield of BAP, about 15-20%.

Synthesis of NAPA Piperazine Derivatives and its Protein Conjugates

Example 5: Synthesis of BCP and BAP

21.535 g of piperazine (0.25 mole), 77 mL triethyl amine (TEA) (0.55 mole) and 300 mL dichloromethane was placed into a 1000 mL flask. 34.81 mL chloroacetonitrile (0.55 mole) was mixed with 100 mL dichloromethane and then added into the piperazine solution with stirring. The reaction solution was stirred at room temperature for 16 hr resulting in the precipitation of a lot of solids. 100 mL HCl (1N) solution was added to extract the product (the reaction solution was extracted three times at least, 100 mL x 3). The aqueous HCl solution was washed with 50 mL dichloromethane twice. The solution pH

was adjusted to 11-12 with sodium hydroxide solution (3N). The solid was collected by filtration and washed with water and dried under vacuum to afford 20 g BCP in about 50% yield. Nuclear magnetic resonance (NMR) was used for the structural determination of the compound, with the following results: ^{1}H NMR (400MHz, $CDCl_3$) δ ppm 2.67 (8H, s), 3.55 (4H, s).

A high-pressure bomb, a commonly used metal vessel structured to withstand very high pressure, was charged with 20 g of bis (cyanomethyl) piperazine (BCP) (0.122 mole); then about 2-3 g Raney nickel catalyst was suspended in 25 mL of 95% ethanol. An additional 25 mL of ethanol was used to rinse the catalyst. The bomb was closed and about 15 g (0.882 mole) of liquid ammonia was introduced from a cylinder. Hydrogen was then admitted at tank pressure (1500 lb), and the temperature was raised to 90°C. When the hydrogen was no longer absorbed after about 2-3 hr, the heater was shut off and the bomb allowed to cool. The hydrogen and ammonia were allowed to escape, and the contents of the bomb were rinsed out with two 50 mL portions of 95% ethanol. The catalyst was filtered and then the solvent was removed by rotary evaporation. The resulting brown oil was further purified by vacuum distillation (70-80°C/5 mmHg) to afford about 4.2 g of BAP in about 21% yield. The total yield of BAP from piperazine was about 10%. The NMR results follow: ^{1}H NMR (400MHz, DMSO-d_6) δ ppm 1.87 (2H, s), 2.23-2.25 (2H, m), 2.37 (2H, m), 2.58-2.71 (6H, m), 3.66 (H_2O, s).

Example 6: Synthesis of succinimidyl p-acetamidyl benzoate (NHS-ester II, *see* sequence III above)

5.375 g of p-acetamidyl benzoic acid (0.03 mole), 8.453 g of DSC (0.033 mole) and 150 THF was placed into a flask. The mixture was stirred at room temperature over 30 minutes. 5 mL triethyl amine was added and then the reaction was stirred at room temperature for 16 hr. The solvent was removed by rotary evaporation. The resulting solid was washed with water three times (under vacuum filtration) to remove side products. The solid was dried under vacuum to yield about 6.0 p-acetamidyl benzoate (NHS-ester II), a 72% yield. The NMR results follow: ^{1}H NMR (400MHz, DMSO-d_6) δ ppm 2.12 (3H, s), 2.89 (4H, s), 3.36 (H_2O, s), 7.84 (2H, m), 8.05 (2H, m).

Example 7: Synthesis of acetyl procainamide piperazine derivative (APP) and APP-acid.

1.69 g of purified BAP and 10 mL THF were placed into a 25 mL flask. 0.687 g of succinimidyl p-acetamidyl benzoate was dissolved into 29 mL THF. The second solution was added into the BAP solution slowly with stirring. The reaction was stirred and monitored by thin layer chromatography (TLC) (100% ethyl acetate as TLC solvent) until no succinimidyl p-acetamidyl benzoate could be detected. A lot of solid was formed. The solvent was then removed by pipette. The solid was washed with ethyl ether three times (10 mL x 3) to remove excess BAP. The solid was dried under vacuum to yield 66% of product (APP, 550 mg).

166.5 mg of APP and 5 mL dimethylformamide (DMF) were placed in a 50 mL flask, then 69.56 μL triethyl amine was added. 50 mg of succinic anhydride was dissolved in 2 mL DMF and added slowly into APP solution with stirring. The reaction was stirred at room temperature for 1 hr. 20 mL ether (Et_2O) was added and the reaction mixture placed in a freezer overnight to precipitate the product (APP-acid). The solvent was removed by pipette and washed with Et_2O twice (5 mL x 2). The remaining solid was dried under vacuum to afford about 100 mg APP-acid in 46% yield.

Example 8: Synthesis of NAPA Protein Conjugates

a. Synthesis of APP-OBt ester

1. 60.00 mg (0.138 mmole) APP-acid and 16.54 mg (0.152 mmole) HOBt were weighed and placed into a 2 mL vial. A magnetic stirrer bar was added. 600 μL dry DMF was pipetted into the vial to dissolve APP-acid and hydroxybenzotriazole hydrate (HOBt).

2. 28.54 mg (0.0.138 mmole) of dicyclohexyl carbodiimide (DDC) was weighed and added into the above solution with stirring.

3. The reaction was stirred at room temperature for 2-3 hr. The activated acid was then ready to couple with proteins.

b. Synthesis of APP-protein Conjugates

Protein Solutions

A. Ovalbumin in 0.15 M $NaHCO_3$

1. 0.315 g of sodium bicarbonate was weighed and added into a 25.0 mL volumetric flask then dissolved with deionized-water to exactly 25.0 mL.

2. 50 mg of ovalbumin was dissolved in 8 mL of 0.15 M $NaHCO_3$ (sodium bicarbonate) in a 16 mL, screw-capped vial with a magnetic stirrer bar.

B. KLH water solution

50 mg of KLH was dissolved in 8 mL deionized water in a 16 mL centrifuge tube by stirring gently overnight in a cold room (4°C). The tube was centrifuged. The supernatant solution was poured into a 16 mL, screw-capped bottle (to be used as a reaction vessel for the conjugate synthesis) and stored in the refrigerator.

C. APP-Conjugation

1. 300 μL each of the above APP-OBt DMF solution from step a above was pipetted into the KLH water (2.B) and ovalbumin buffer solution (2.A), respectively, with stirring. The reactions were stirred gently at room temperature for 10 min. then placed in a cold room at 4°C overnight.

2. The two APP conjugated protein solutions above were dialyzed against three changes of deionized water, then against three changes of phosphate buffered saline (PBS) buffer (using dialysis tubing with a 6-8,000 MW cut-off range for the ovalbumin conjugate).

Synthesis of Procainamide Piperazine Derivatives and its Protein Conjugates

Example 9: Synthesis of BOC-BA and BOC-SB

a. Make BOC-BA

1. 3.425 g of amino benzoic acid (ABA) and 1.5 g sodium hydroxide were weighed and placed into a 100 mL flask.

2. 25 mL water was added with stirring.

3. 5.995 g of di-t-butoxy dicarbonate (DBDC) was added.

4. The reaction was stirred at room temperature overnight.

5. The solution was acidified with 1N HCl to pH 4-5 (about 37.5 mL 1N HCl is needed).

6. The product was extracted with ethyl acetate three times (3 x 25 mL).

7. The ethylacetate (EtOAc) solution was washed with water three times (3 x 25 mL).

8. The EtOAc solution was dried with $MgSO_4$.

9. The solvent was removed by rotary evaporation.

10. The product was weighed (W) and the yield was calculated using the formula

$$Y = (W/5.01) \times 100\%.$$

b. Synthesis of BOC-SB

1. 3.555 g of BOC-BA (0.015 mole) was weighed and dissolved into 50 mL acetonitrile.

2. 4.224 g of DSC (0.015 x 1.1 mole) and 5.22 mL TEA (0.015 x 2.5 mole) were added to that mixture.

3. The reaction was stirred at room temp. for 2-3 hr. (The reaction was checked with 50% EtOAc and 50% hexane).

4. The solvent was removed.

5. The purity of the product was checked by TLC (column chromatography was used from time to time to purify the product)

6. The product (W) was weighed and the yield calculated using the formula

$$Y = (W/5.925) \times 100\%.$$

The NMR results follow: ^1H NMR (400MHz, DMSO-d_6) δ ppm 1.50 (9H, s), 2.88 (4H,s), 3.34 (H_2O, s), 7.71 (2H, m), 8.00 (2H,m).

Example 10: Synthesis of BOC-PP and BOC-PP-NHS Ester

a. Synthesis of BOC-PP (t-Butoxyl Carbamyl Procainamide Piperazine Derivative)

1. 0.688 mg of BAP was dissolved into 10 mL THF.

2. 334 mg of BOC-SB was dissolved in 15 mL THF.

3. The solution of step 2 (BOC-SB) was added slowly into the solution of step 1 (BAP) with stirring. Some solid precipitated after 5-10 minutes.

4. The reaction was stirred at room temperature over 30 minutes.

5. The solvent was removed by pipette. The solid was washed with ethyl ether three times (10 mL x 3) to remove excess BAP.

6. The solid was dried under vacuum to yield 87% of product (BOC-PP, 340 mg).

BOC-PP is very hygroscopic, and should be stored in the desiccator.

b. Synthesis of BOC-PP-NHS Ester

1. 107.92 mg (0.276 mmole) BOC-PP was weighed and placed into a 4 mL vial. A magnetic stirrer bar was added. 500 μL dry DMF and 40 μL TEA were pipetted into the vial to dissolve BOC-PP.

2. 27.6 mg (0.276 mmole) of succinic anhydride was dissolved into 100 μL DMF, and added into the solution of step 1 with stirring; the vial was rinsed with 100 μL DMF solvent and added to the solution to ensure quantitative transfer.

3. The reaction was stirred at room temperature for 1 hr.

4. 77.78 mg DSC (0.152 mmole) was weighed and added into the above reaction solution followed by the addition of 56.0 μL TEA with stirring.

5. The reaction was stirred at room temperature for 2 hr.

Example 11: Synthesis of Procainamide protein Conjugates

a. Protein Solutions

A. Ovalbumin in 0.15M $NaHCO_3$.

1. 0.630 g of sodium bicarbonate was weighed and added into a 50.0 mL volumetric flask then dissolved with deionized-water to exactly 50.0 mL.

2. 100 mg of ovalbumin was dissolved in 16 mL of 0.15 M $NaHCO_3$ (sodium bicarbonate) into a 40 mL, screw-capped vial with a magnetic stirrer bar.

B. KLH water solution

100 mg of KLH was dissolved in 16 mL deionized-water in a centrifuge tube by stirring gently overnight in a cold room at 4°C. The tube was centrifuged and the supernatant solution poured into a 40 mL, screw-capped

bottle (to be used as reaction vessel for the conjugate synthesis) and stored in the refrigerator.

b. Conjugation of BOC-PP-NHS with Proteins

400 μL of the above BOC-PP-NHS DMF solution from Example 6 b.5 (0.138 mmole of BOC-PP-NHS) was pipetted into the KLH water and the ovalbumin buffer solution with stirring. The reactions were stirred gently at room temperature for 10 min. then stored in a cold room (4°C) overnight.

c. Deprotection of BOC-group and Purification of Conjugates

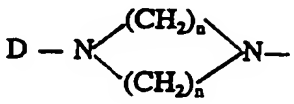
1. The two BOC-PP-NHS conjugated protein solutions were dialyzed against three changes of deionized water.

2. The two protein solutions were lyophilized under vacuum (freeze-dry) to get the protein solid. To each protein was added 5 mL CH_2Cl_2, followed by 5 mL trifluoroacetic acid. After stirring 5 min, solvent was concentrated under vacuum and the residue redissolved into 16 mL PBS buffer solution. The resulting cloudy solutions were dialyzed against three changes of deionized-water and three changes of PBS buffer solution. (using dialysis tubing with a 6-8,000 MW cut-off range).

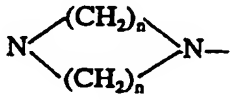
The reactive derivatives of the present invention provide novel compounds for conjugating the drug or other compound having a dialkyl amino group, to a protein or other carrier compound. A variety of drugs having dialkyl amino groups can benefit from the derivation of the present invention. The procedure for derivatizing two different types of drugs to produce the reactive derivatives of the present invention have been described. Those skilled in the art will recognize that other methods for derivatizing other dialkyl amino compounds may be used and may vary for different compounds, but the precise derivatization technique is within the skill of the art. The procedures for conjugating the terminal N of the reactive piperazine-like derivative or the functional group of a bifunctional spacer to the carrier compound will differ for different drug and carrier compound combinations, but are also well within the skill of those in the art.

What we claim is:

1. A composition for conjugation to a carrier compound comprising:



wherein D is a compound having in its underivatized structure a dialkyl amino group, which dialkyl amino group has been replaced with the



residue of the composition, and n is an integer greater than or equal to 1.

2. The composition recited in claim 1 wherein D is a drug selected from the group consisting of lidocaine, procainamide, N-acetyl procainamide, dispyramide, chloroquine, diphenhydramine, methadone, imipramine, desipramine, amitriptyline, noxiptilin and nortriptyline.

3. The composition recited in claim 1 wherein the terminal nitrogen is bound to a carrier compound.

4. The composition of claim 3 wherein the carrier compound is a proteinaceous material.

5. The composition of claim 3 wherein the carrier compound is bound to a solid support.

6. The composition of claim 5 wherein the solid support is a polymer particle.

7. The composition recited in claim 1 further comprising a bifunctional spacer bound to the terminal nitrogen.

8. The composition of claim 7 wherein the bifunctional spacer has a first functional end bound to the terminal nitrogen of the piperazine like derivative and a second functional end bound to a carrier compound.

9. The composition of claim 8 wherein the bifunctional spacer is selected from the group consisting of cycloanhydrides, bis-N-succinimidyl derivatives and dialdehydes.

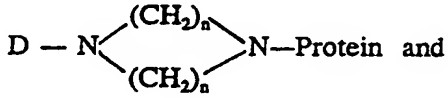
10. The composition of claim 8 wherein the carrier compound is a proteinaceous material.

11. The composition of claim 8 wherein the carrier compound is bound to a solid support.

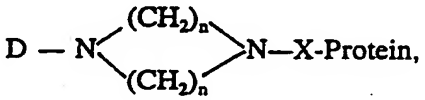
12. The composition of claim 11 wherein the solid support is a polymer particle.

13. An immunogen comprising:

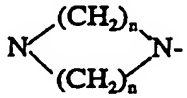
a composition selected from the group consisting of



and



wherein D is a drug having in its underivatized structure a dialkyl amino group which dialkyl amino group has been replaced by the



derivative of the composition, n is an integer greater than or equal to 1, and X is a bifunctional spacer.

14. The immunogen of claim 13 wherein the drug is selected from the group consisting of lidocaine, procainamide, N-acetyl procainamide, dispyramide, chloroquine, diphenhydramine, methadone, imipramine, desipramine, amitriptyline, noxiptilin and nortriptyline.

15. The immunogen of claim 13 wherein the bifunctional spacer is selected from the group consisting of cycloanhydrides, bis-N-succinimidyl derivatives and dialdehydes.

16. The immunogen of claim 13 wherein n is an integer from 1 to 3.

17. The immunogen of claim 13 wherein the Protein is selected from the group consisting of ovalbumin, keyhole limpet hemocyanin and bovine serum albumin.

18. A particle reagent comprising:

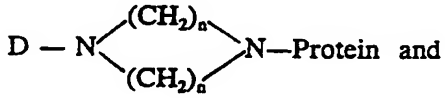
(a) a polymer particle having an inner core and an outer shell wherein the inner core is a polymer having a refractive index of not less than 1.54 as measured at the wavelength of the sodium D line, and wherein the outer shell is a polymer of

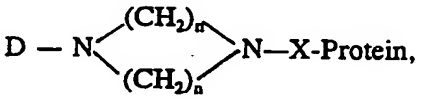
(i) an ethylenically unsaturated monomer having a functional group capable of reacting with a nucleophilic compound of biological interest,

(ii) optionally, other ethylenically unsaturated monomers in an amount sufficient to produce water insoluble polymer particles, and

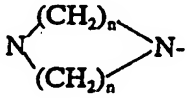
(iii) not more than 10 parts by weight of the outer shell of the monomers of the inner core, the outer shell being formed by polymerization in the presence of the inner core, covalently bound to;

(b) a composition selected from the group consisting of





wherein D is a drug having in its underivatized structure a dialkyl amino group which dialkyl amino group has been replaced by the



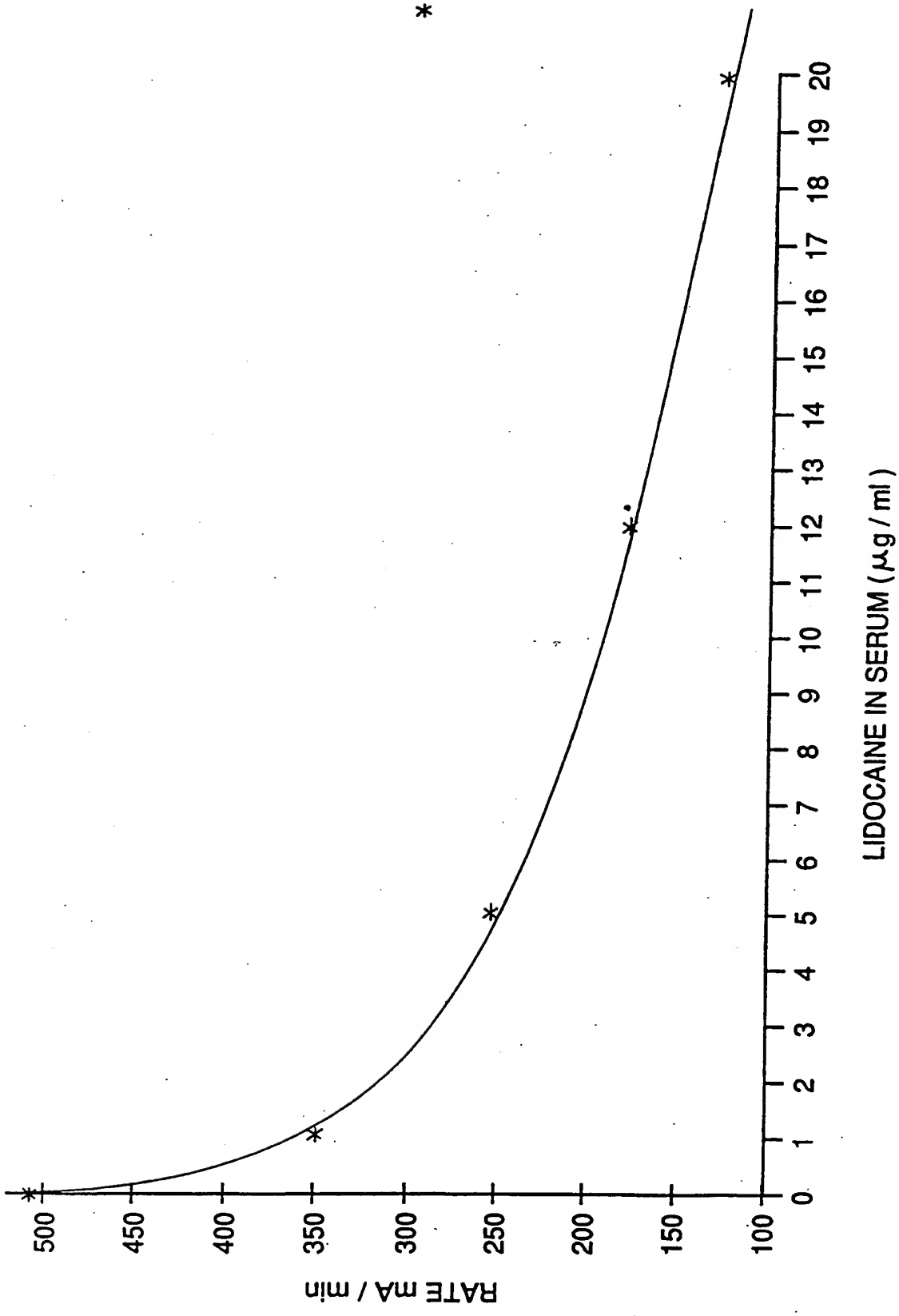
derivative of the composition, n is an integer greater than or equal to 1, and X is a bifunctional spacer.

19. The particle reagent of claim 18 wherein the drug is selected from the group consisting of lidocaine, procainamide, N-acetyl procainamide, dispyramide, chloroquine, diphenhydramine, methadone, imipramine, desipramine, amitriptyline, noxiptilin and nortriptyline.

20. The particle reagent of claim 18 wherein the bifunctional spacer is selected from the group consisting of cycloanhydrides, bis-N-succinimidyl derivatives and dialdehydes.

21. The particle reagent of claim 18 wherein n is an integer from 1 to 3.

22. The particle reagent of claim 18 wherein the carrier compound is selected from the group consisting of bovine serum albumin, polyethylene polyamine and human serum albumin.


RATE mA / min
500
450
400
350
300
250
200
150
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
LIDOCAINE IN SERUM (µg / ml)

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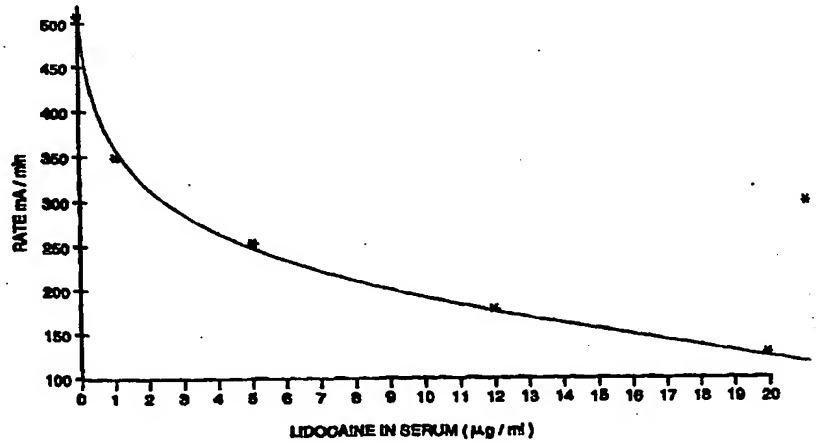

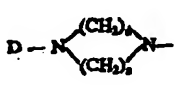

(a)

(57) Abstract

The invention provides a reactive derivative of dialkyl amino compounds, particularly dialkyl amino drugs, for facilitating the conjugation of the drug, directly or through a bifunctional spacer, to a carrier compound such as proteinaceous materials. The derivative has formula (a) wherein D is the drug and n is an integer greater than 1, and preferably equal to 2. The drug derivative carrier conjugate can be used as an immunogen for production of antibodies specific to the drug. Additionally, the conjugate can be coupled to a solid support, such as a polymer particle, for use as a particle reagent in immunoassays specific to the drug.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D295/14 A61K7/48 C07D295/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 4 558 129 A (KLUGE ARTHUR F ET AL) 10 December 1985 see claims ---	1-22
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1

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1997	12.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Sánchez García, J.M.

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Inter nal Application No
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